FINAL REPORT

ARB Contract A9-076-31

NEW APPROACH FOR DETECTING HEALTH HAZARDS OF NO_2 INHALATION

Period: 1/21/79 - 5/30/81

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1. ABSTRACT

On the basis of preliminary observations which suggested that inhalation of ambient levels of NO_2 may facilitate blood borne cancer cell metastasis to the lungs, more extensive studies were undertaken to investigate this aspect further. Since increased blood borne cancer cell metastasis to lungs may reflect injury to the pulmonary microcirculation or alterations in the immune system or even both, the development of metastases could serve as a very meaningful biological indicator for harmful NO_2 effects. Moreover, it would have direct relevance to human health since many humans, particularly cancer patients, harbor cancer cells in their circulation. Experiments were designed, using a mouse model, to test ambient level NO_2 inhalation effects on the frequency of blood borne cancer cell metastasis development. To date, five experiments have been carried out, two of which were preliminary studies and the other three were major experiments. The two preliminary experiments (M125, M130) were intended to finalize the methodologies and involved 26 and 40 animals respectively and both were carried out at 0.8 ppm NO_2 exposure. The three major experiments (M128, M137, M139) involving 270 animals were carried out at 0.8 ppm, 0.3 ppm and 0.5 ppm NO_2 exposure respectively. There were three groups of animals in each experiment (filtered air control, NO_2 exposed, and vivarium ambient air control) with 30 animals per group. The results have indicated that the 0.8 ppm NO_2 exposed animals developed the highest frequency of melanoma nodules when compared to control animals who inhaled filtered NO_2 free air. Similar results were also obtained with 0.3 ppm exposure for 12 week period but with a lower level of significance. The animals residing in ambient vivarium air also showed more melanoma nodules in their lungs than did the controls. However, exposure for 8 weeks to 0.5 ppm did not reveal significant differences between the three groups and the latter may indicate that the length of exposure to ambient level pollutants is important. Thus, the data obtained from the major portion of this study indicates that the inhalation of ambient levels of NO_2 or ambient vivarium air, under the described experimental conditions, facilitates blood borne cancer cell metastasis to the lungs. Further studies are warranted since other exposure conditions need to be evaluated and the question arises as to whether similar events may take place in human urban populations.

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3. DISCLAIMER

"The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products."

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6. SUMMARY AND CONCLUSIONS

The major objective of the experiments was to determine if the inhalation of ambient level NO_2 could facilitate blood borne cancer cell dissemination to lungs. In order to test this, experimental animals (mice) were exposed to ambient levels of NO_2 (0.3, 0.5 or 0.8 ppm), while the control and the room control animals inhaled NO_2 free or ambient vivarium room air respectively. Following the exposure period, cancer cells (B16 mouse melanoma cells) were infused into the blood stream of the animals and three weeks later the lungs were examined for the development of cancer nodules. It was reasoned that any damage incurred by inhalation of NO_2 to the lungs, to the immune system, or both should cause increased blood borne cancer cell spread or metastasis to the lungs of exposed animals. Thus, the frequency of metastases development when compared between control and exposed animals would serve as an indicator for harmful NO_2 effects. The end result, which is cancer cell metastasis, is a very meaningful biological event and has direct relevance to human health since the probability exists that one in four individuals will develop cancer during their life time. Moreover, cancer cells in peripheral blood can be found not only in cancer patients but in individuals clinically free of cancer.

The results from the experiments performed, where 0.3, 0.5 and 0.8 ppm of NO_2 were tested, have indicated that the animals exposed for 12 weeks to 0.8 or 0.3 ppm of NO_2 developed a significantly higher number of cancer nodules in their lungs when compared to lungs of filtered air controls. Of further significance is the finding that animals inhaling ambient vivarium air developed more lung metastasis than the filtered air controls. It is of interest that 0.3 ppm NO_2 exposure had the same effect as the ambient vivarium air exposure. Considering that most of the time vivarium air has levels of NO_2 lower than 0.1 ppm, the observed results may be due to a combination of several low concentration pollutants present in ambient air. The exposure for 8 weeks to 0.5 ppm of NO_2 did not show significant differences in the development of melanoma nodules and the latter findings may indicate that the length of the exposure at ambient levels plays an important role. Additional findings included changes of body and spleen weights in the exposed groups of animals, indicating the presence of systemic effects.

To date, the data has indicated that inhalation of ambient levels of NO_2 or ambient vivarium air for a period of 10 weeks or longer facilitates blood borne cancer cell metastasis to the lungs. The mechanisms are not clear but it most likely reflects damage to the lung capillary bed, the immune system or to both. Further studies are needed to investigate other exposure conditions in order to establish a more precise relationship between the inhalation of NO_2 and the facilitation of blood borne cancer cell metastasis. The information obtained may be of assistance in developing health safety standards. Most importantly, the findings of these experiments raise the question as to whether similar events are taking place in urban human populations, a question which warrants immediate attention.

7. RECOMMENDATIONS

The experiments described in this report indicates that under certain conditions inhalation of ambient levels of NO_2 (0.3 and 0.8 ppm) or ambient vivarium air facilitates blood borne cancer cell spread or metastasis to the lungs. However, only one specific condition has been tested in these experiments, that is, the NO_2 exposure occurred prior to the infusion of cancer cells into the blood stream. Other experimental conditions have to be tested to determine the full impact of NO_2 inhalation hazards with respect to blood borne cancer cell metastasis. Moreover, the findings raise the possibility that similar events may take place in the human urban population. Thus, it is recommended to test the frequency of cancer cell metastasis in an experimental system under the following conditions: 1) NO_2 exposure alternated with clean air inhalation; 2) NO_2 exposure immediately after cancer cell introduction into the blood stream; 3) NO_2 exposure while cancer cells are in circulation; 4) to establish the shortest effective exposure period at moderate ambient NO_2 levels (0.4 ppm) which would result in an enhancement of cancer cell metastasis; 5) to establish the mechanism responsible for the enhancement; 6) to evaluate other oxidants such as O_3, or an O_3 and NO_2 combination using the same parameters. In addition, it is recommended to conduct epidemiological studies to determine the frequency of cancer metastases development in cancer patients who are residing in or have lived in a polluted urban environment.

8. BODY OF REPORT

Introduction

a. Scope and purpose of the project, general background of the project.

The presence of pollutants in the environment, especially those with carcinogenic properties has been of great concern to environmental health scientists. In view of this, many studies have been directed toward the identification of cancer causing agents in the environment (1-4). However, the problem of cancer involves not only the development and presence of neoplastic cells at a primary site, but also the ability of these cells to migrate, seed, proliferate and develop secondary cancer masses or metastases in distant organs and tissues (5,6). Considering the fact that a significant segment of the population in the United States is already affected by cancer together with the probability that one in four individuals will develop cancer (7), the question arises as to the role environmental pollutants play not only in the causation of cancer or carcinogenesis, but in the dissemination of cancer cells and the development of metastases. It is well established that most cancer patients have circulating cancer cells (8,9) and in some instances cancer cells have been demonstrated in the circulation of patients who are clinically free of cancer (10,11). Circulating cancer cells are also found in the peripheral blood of tumor bearing animals (12). Moreover, there are several known conditions which may favor the development of cancer cell metastases from circulating cancer cells and include the following: 1) immune suppression, 2) capillary endothelial cell alterations, 3) cancer cell interactions with components of blood clotting mechanisms and 4) tissue damage in general (4,5,13-17). Importantly, it is known that several of the above mentioned conditions occur as a result of nitrogen dioxide (NO_2), a common air pollutant, inhalation (18-21) and thus one may expect that NO_2 inhalation could facilitate or enhance circulating cancer cell metastasis. Most pertinent to this project are the studies showing changes in lung capillaries and in the cells of the lung itself due to NO_2 exposure (22-24) since the capillary cell injury and trauma in general have been associated with enhanced blood borne cancer cell lodgement, proliferation and metastases development (11,15,22). Thus, studies were designed to investigate the effects

of NO_2 inhalation on the frequency of blood borne cancer cell metastases development in lungs of experimental animals. It was intended to determine if NO_2 could act as a facilitator of cancer cell dissemination by affecting the host in a non-carcinogenic manner. Besides our preliminary observations (26) there are no other reported studies where NO_2 inhalation effects have been investigated with respect to circulating cancer cells and metastases development. In this study the frequency of blood borne cancer cell metastasis to the lungs is used as an indicator for adverse NO_2 effects. We consider this new approach and the studies outlined in this report to be highly relevant to human health and to the setting of air quality standards.

8. DESIGN, MATERIALS, AND METHODOLOGY

The test model involved B16 mouse melanoma cells, which were grown in vitro (27). Cell suspensions were prepared for use in experiments and 100,000 cells were infused into the circulation of C57 B1/J6 mice. These cells will spread from circulation to lungs and will develop in the lungs easily recognizable black nodules which can be quantitated microscopically. The nodule growth progresses to kill the animal. By comparing the frequency of melanoma nodule development in NO_2 exposed and control animals, the role of NO_2 in the facilitation of metastases development will be established. Experiments were designed to test three different ambient concentrations of NO_2, i.e. 0.3 ppm, 0.5 ppm and 0.8 ppm. During the contract period, two preliminary and three major experiments were carried out

Preliminary Experiments

Experiment M125-Lung metastases development following 12 weeks of 0.8 ppm NO_2 exposure

This experiment utilized 26 C57 B1/J6 mice with 13 animals in the control and 13 animals in the exposed group. Animals were exposed for 12 weeks to 0.8 ± 0.05 ppm NO_2 for 7 hours a day for 5 days per week for a total of 420 hours. The nitrogen dioxide gas was introduced into the exposure chamber via the air intake by a method which has been in use in this laboratory for the past several years (28). The level of the gas was continuously monitored with a Teco chemiluminescence NO_2 analyzer and a Beckman analyzer utilizing Saltzman fluid. In addition, at least two weekly NO_2 gas level checks were performed with a fritted bubbler employing the technique of Saltzman (29). The control animals received filtered NO_2-free air. After the exposure period, each animal was infused intravenously via the tail vein with 100,000 B16 F10 R1 melanoma cells to study blood borne cancer cell metastasis. Following the injections, the animals were returned to their respective environmental chambers and were treated as before for an additional 19 days. After this time period, the animals were killed and the following determinations were made: 1) body weights; 2) spleen weights; and 3) number of melanoma nodules per lung. Lung and spleen tissues were also taken for histopathological studies.

Experiment M130 - Subcutaneous melanoma growth following 7 days of 0.8 ppm NO_2 exposure

This experiment was slightly modified from the previous one in that subcutaneous tumor growth and spread, instead of blood borne cancer cell spread and dissemination, was studied. Two groups of animals were employed, the experimental group received 0.8 ± 0.0 ppm NO_2 and the control group received filtered ambient air. Animals were kept in the appropriate environmental chambers for a period of seven days. After this time period, they were subdivided into four groups with 10 mice per group. Each group received 50,000 melanoma cells transplanted subcutaneously on the antero-lateral side. Groups X and C were returned to the environmental chambers after transplantation while groups RX and RC were kept in ambient vivarium air and became the room controls to the corresponding experimental and filtered air controls. This protocol was established to determine the effects, if any, on tumor development inside and outside the chambers.

The Major Experiments

Experiment M128 - Lung metastases development following 12 weeks of 0.8 ppm NO_2 exposure

The main objectives of this experiment was to determine if the inhalation of NO_2 (0.8 ± 0.05 ppm) influences the frequency of blood borne cancer cell metastasis to the lungs. There were three groups of animals; 1) experimental (NO_2 exposed); 2) filtered air control; and 3) vivarium ambient air control. Each group originally had 30 animals. The NO_2 exposed animals were designated as group "X", the filtered air group as "C" and the ambient air control as "CR". During the course of the experiment several animals of each group engaged in fighting and even though they were separated from the rest of the animals, they had developed extensive back and tail lesions at the conclusion of the experiment and were not incorporated in this study. Thus, the final number of animals per group was decreased, i.e., 23 controls, 24 exposed, and 19 ambient air controls. The exposure period in this experiment was 12 weeks and the X group of animals were exposed for eight hours per day, five days per week. After 12 week period, all three groups of animals received tail vein infusions of 5×10^4 melanoma

cells. They were then returned to ambient air of the vivarium for three weeks. The following determinations were made after sacrificing the animals: 1) body weights; 2) spleen weights; and 3) number of melanoma nodules per lung. Lung and spleen tissues were also taken for histopathological studies.

Experiment M137 - Lung metastases development following 12 weeks of 0.3 ppm NO_2 exposure

The NO_2 exposure level for this experiment was 0.3 ± 0.05 ppm. The remainder of the protocol in this experiment was the same as for experiment M128.

Experiment M139 - Lung metastases development following 8 weeks of 0.5 ppm NO_2 exposure

The NO_2 exposure level for this experiment was 0.5 ± 0.05 ppm and the length of exposure was 8 weeks for a total of 280 hours. Some animals died during the experiment and the final number of animals suitable for the study were 29 filtered air controls, 23 NO_2 exposed, and 26 ambient air or room control. The remainder of the protocol was the same as for the other major experiments.

Data Analysis

All data was analyzed by computer assisted parametric or nonparametric methodologies. The body and spleen weights were usually evaluated by Student's t-test on basis that they have normal distribution. The statistics for the melanoma nodule frequency in the lungs were obtained from t-tests for the preliminary experiments and the major experiments were analyzed by t-test as well as Willcoxon 2-sample and Kruskal-Wallis methods. The latter nonparametric methods have advantage over parametrics since they do not depend upon stringent assumptions and have no set minimum sample size. The statistics from parametric and nonparametric analyses were presented for comparison.

c. RESULTS

Experiment M125 - Lung metastases development following 12 weeks of 0.8 ppm NO_2 exposure.

The results of experiment M125 indicated the following: 1) the NO_2 exposed animal group had a lower mean body weight than the control animals ($p < 0.05$); 2) spleen weights were lighter in exposed animals ($p < 0.1$); and 3) the mean number of melanoma nodules per lungs was slightly higher in control animals (42 vs 34), however, the difference was not significant. Several of the nodules were fused with other smaller nodules indicating that the injected number of cells could be reduced to produce smaller number of nodules.

Experiment M130 - Subcutaneous melanoma growth following 7 days of 0.8 ppm NO_2 exposure.

The first palpable melanoma nodules were noted 13 days posttransplantation. Measurements were taken weekly and the final measurements of the nodules were taken at 21 days posttransplantation since after this time period animals began to die and the numbers in each group became too small to be of value for tumor size comparisons. The tumor index (width x length) was established from 21 day measurements. The index showed slightly larger tumors in C group ($p < 0.1$) and CR group ($p < 0.05$) versus X and XR groups respectively. These results may indicate general growth suppression in NO_2 exposed animals. The majority of control animals also died sooner than the exposed animals from the tumor burden. There were no differences observed in melanoma growth rate within the treatment groups of animals comparing the tumor development inside or outside the environmental chambers. With respect to metastasis, there were no metastases detected in the lungs or other organs of the animals. The body and spleen weights were not determined during the course or at the end of this experiment.

Experiment M128 - Lung metastases development following 12 weeks of 0.8 ppm NO_2 exposure.

The results for experiment M128 are presented in Table 1. The NO_2 exposed animals show a higher frequency of melanoma nodules per lung than do the controls. The Student's t-test analysis showed that the difference is highly significant with $p < 0.0025$. In comparing the Vivarium ambient air control,

the animals inhaling ambient air showed a higher frequency of melanoma development ($p < 0.05$). Statistical analysis by nonparametric method did not show significant differences between filtered air and ambient air (Vivarium room air) groups. The discrepancy may be due to different sensitivity of the test, the size of the sample or distribution of tumor nodules. However, the trend towards increased number of melanoma nodules in animals of ambient air group is still there (Table 6). Evaluation was also carried out with respect to melanoma nodule distribution in different lobes of the lung and the results are presented in Table 2. The data indicate that the left lobe and the right upper lobe develop more nodules from the blood borne cancer cells. The comparison of percent spleen weights among the different groups showed no significant differences.

Experiment M137 - Lung metastases development following 12 weeks of 0.3 ppm NO_2 exposure.

A summary of results for experiment M137 is presented in Table 3. The number of nodules was evaluated with respect to lung and also with respect to the lobes of individual lungs. The NO_2 exposed animals showed a higher frequency of melanoma nodules per lung and per lobe than did the filtered air controls with $p = 0.05$ and $p < 0.01$ respectively. In comparing the filtered air control versus the vivarium ambient air control, the vivarium air controls had a higher frequency of melanoma nodules per lung ($p < 0.05$) and also a higher frequency per lobe ($p < 0.01$). Another finding of interest was the increase in percent spleen weights in the exposed animals when compared to control animals ($p < 0.005$). A similar increase was observed in the vivarium ambient air controls ($p < 0.01$), Table 4. There were no body weight differences observed among the groups.

Experiment M139 - Lung metastases development following 8 weeks of 0.5 ppm NO_2 exposure.

A summary of results for experiment M139 is presented in Table 5. It can be seen that no significant differences were detected between the three groups of animals after 8 weeks of NO_2 exposure. This was true when the data was analyzed with respect to each lung or with respect to individual lobes within the lung. There were no body weight differences observed among the groups and the spleen weight data was not collected.

A summary of results from all three of the major experiments is presented in Table 6 and it can be seen that ambient level NO_2 exposures for 12 weeks facilitated melanoma nodule development from circulating cancer cells while 8 weeks of exposure did not. It is also of interest that ambient vivarium room air exposure for 12 weeks facilitated melanoma nodule development in experiment M137 and the same trend can be noted in experiment M128.

Histopathology

Limited microscopic examination of randomly selected samples from all experiments was carried out. The morphology of melanoma nodules was consistent with malignant growth, i.e. it showed cell proliferation, and invasion and destruction of lung parenchyma. The morphology and staining properties of the malignant cells was also consistent with those of malignant melanocytes. The identity of cells was further confirmed by electron-

microscopy which revealed melanosomes in different stages of development in all of the cells examined. The melanoma nodules showed very limited inflammatory response, consisting of few lymphocytes and macrophages.

d. DISCUSSION

The preliminary experiments of this project permitted evaluation of in vivo viability and the growth potential of B16 F10 R1 melanoma cells under the set experimental conditions. Utilizing this information and our previous experience with these cells it was possible to establish that the infusion of 5×10^4 melanoma cells into the blood stream of experimental animals could be used as a probe to delineate NO_2 exposed animals since they developed melanoma nodules with higher frequency in their lungs than did the controls. The frequency of lung metastases development from the infused cancer cells was utilized as an indicator for the extent of NO_2 induced alterations in the host. These are the first reported projects where this new approach has been utilized to detect harmful NO_2 effects.

The major findings from the experiments described in this report are the increased frequency of melanoma nodule development in the lungs of NO_2 exposed animals and animals exposed to presumably polluted vivarium room air. This of course is not surprising since vivarium air is only filtered through conventional air conditioners and one can expect a variety of pollutants similar to those found in outside ambient air to be present as well. The most significant differences with respect to the number of melanoma nodules developing in the lungs was observed after 0.8 ppm of NO_2 exposure for 12 weeks. It is of interest that inhalation of 0.3 ppm of NO_2 produced the same results as inhalation of vivarium room air which may mean that inhalation of a concentration of 0.3 ppm of NO_2 is comparable to inhalation of ambient room air which presumably has a variety of lower concentration air pollutants. The room air in our experiments was monitored only for NO_2 which was always below 0.1 ppm.

It is important to point out that in the major experiments of this project, the NO_2 exposure always preceded the introduction of cancer cells into the blood stream and thus these experiments have tested only this particular condition. Only one preliminary experiment has been carried out where cancer cells were introduced into the blood stream and the animals were kept under exposure conditions instead of vivarium air for another 19 days. In the latter experiment there were no significant differences in the number of melanoma nodules developing in the exposed or control animal lungs. The smaller number of animals used in the latter experiment did not permit any conclusions

to be drawm, but the possibility exists that circulating cancer cells at 0.8 NO_2 exposure may not be as capable of surviving as cells at lower exposure levels. Particularly since it is known that NO_2 reaction products (NO_2^-, NO_3^-) exist in blood of NO_2 exposed animals and may react with circulating cellular components. Thus the inhalation of 0.8 ppm of NO_2 may be detrimental not only to certain lung cells but also to blood cells as well as circulating cancer cells. Any alterations in cancer cell surface properties or motility could affect the metastatic potential. If the latter occurred, which was not established in these experiments, an explanation could be provided for finding lower mean number of melanoma nodules (34X vs 42C) in NO_2 exposed group. Even though this difference is not statistically significant ($p < 0.1$), considering the small number of animals employed (9C and 12X), the trend is there and it may have biological meaning. Different circumstances may have played a role in experiment M139 and the length of exposure, not necessarily the dose of NO_2, should be considered. Namely, prolonged exposure to low level ambient air pollutants or low level NO_2 may be more detrimental to the host than exposures of a shorter duration to slightly higher ambient levels of pollutants. For this reason and the fact that other different exposure conditions may exist, there is a great need for expanded studies in this area to investigate different exposure conditions and different oxidants.

With respect to the spleen changes, the only significant change was noted in experiment M137 where the percent spleen weights of NO_2 exposed and Vivarium room air exposed animals were higher than in filtered air controls following 12 weeks of exposure. No definitive conclusions can be drawn but it may be indicative of systemic NO_2 effects. In addition, it points out that NO_2 may affect directly or indirectly organs other than the lung.

Even though these studies are of a limited nature and utilize an animal system, we believe the findings are highly relevant to human health. Observations in humans showing that cancer cells will spread to tissues damaged by trauma or radiation (9,11,15), further emphasize the need for additional investigations relating NO_2 induced tissue alterations with cancer metastasis.

Some epidemiological studies of certain human populations have reported results which indicate an increased frequency of cancer related deaths (30,31) in polluted urban environments, while other studies have not observed this latter correlation (32,33). However, there have not been studies designed to

deal specifically with the frequency of metastases development. Such studies are urgently needed. It should also be mentioned that even though these are the first experiments where inhalation of NO_2 is associated with facilitation of blood borne cancer cell metastasis, there is a study where inhalation of cigarette smoke in an experimental system has been linked with the facilitation of cancer cell metastasis (34). It is anticipated that further studies along these lines should clarify some of the questions raised and the mechanisms which may be involved.

TABLE 1

Experiment M128

12 Weeks of 0.8 ppm NO_2 Exposure

Frequency of Melanoma Nodules Per Lung

Treatment Groups	No. Animals	Mean No. Melanoma Nodules Per Lung ± SD	Student t-test
Filtered air (C)	23	7.4 ± 4.5	C vs X $p < 0.0025$
NO_2; 0.8 ppm (X)	24	19.0 ±15.3	RC vs X $p < 0.025$
Vivarium room (RC) air	19	10.8 ± 7.5	C vs RC $p < 0.05$

TABLE 2

Experiment M128 - 12 Weeks of
0.8 ppm NO_2 Exposure

Distribution of Melanoma Nodules in Lungs

Treatment	No. Animals	Nodules Per Lobe*					Total No. Nodules
		L	RU	RM	RL	C	
Filtered air	23	52	33	30	40	17	172
NO_2; 0.8 ppm	24	115	121	67	94	52	449

*L - Left lobe

RU - Right upper lobe

RM - Right middle lobe

RL - Right lower lobe

C - Cardiac lobe

TABLE 3

Experiment M137 - 12 Weeks of

0.3 ppm NO_2 Exposure

Frequency of Melanoma Nodules

Treatment	No. of Animals	No. of Nodules* Per Lobe ± SD	No. of Nodules* Per Lung ± SD	Student's t-test
Filtered air (C)	29	2.73 ± 2.86	10.07 ± 11.13	C vs X lobe: $p < 0.01$ lung: $p = 0.05$
NO_2; 0.3 ppm (X)	25	3.87 ± 3.67	15.64 ± 13.81	C vs RC lobe: $p < 0.01$ lung: $p = 0.05$
Vivarium room (RC) air	28	3.62 ± 2.83	15.14 ± 11.04	X vs RC lobe: NS lung: NS

NS - No significant difference

* - Mean number of nodules

TABLE 4

Experiment M137 - 12 Weeks of
0.3 ppm NO_2 Exposure
% Spleen Weights*

Treatment	No. of Animals	% SW ± SD	Student's t-test
Filtered air (C)	30	.244 ± .026	C vs X $p < .005$
NO_2; 0.3 ppm (X)	29	.265 ± .025	C vs RC $p < .01$
Vivarium room (RC) air	30	.260 ± .022	X vs RC NS

NS - No significant difference

*$\frac{\text{spleen weight}}{\text{body weight}} \times 100$

TABLE 5

Experiment M139 - 8 Weeks of
0.5 ppm NO_2 Exposure
Frequency of Melanoma Nodules

Treatment	No. of Animals	No. of Nodules* Per Lobe	No. of Nodules Per Lung	P Value
Filtered air	29	2.7	10.7	NS
NO_2; 0.5 ppm	23	2.4	9.3	NS
Vivarium room air	26	3.0	10.9	NS

NS - Not Significant

P - Wilcoxon 2 sample test

* - Mean number of nodules

TABLE 6

FREQUENCY OF PULMONARY METASTASES

EXP.	TREATMENT	NO. ANIMALS	MEAN NO. NODULES PER LUNG	P VALUE
M128	Filtered air	23	7.5	FA vs NO_2 p = 0.0004
12 Weeks	NO_2; 0.8 ppm	24	19.0	
	Ambient Vivarium Air	19	10.8	FA vs AVA NS
M137	Filtered air	29	10.1	FA vs NO_2 p = 0.0542
12 Weeks	NO_2; 0.3 ppm	25	15.6	FA vs AVA
	Ambient Vivarium Air	28	15.1	p = 0.0305
M139	Filtered air	29	10.7	NS
8 Weeks	NO_2; 0.5 ppm	23	9.3	
	Ambient Vivarium Air	26	10.9	NS

P = Wilcoxon 2-Sample Test
FA = Filtered Air
AVA = Ambient Vivarium Air
NS = Not Significant

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10. PUBLICATIONS AND PRESENTATIONS

Utilizing the data which were obtained before the contract was awarded and the data produced by contract research, we have been able to put together three publications which were reviewed by Mr. Westerdahl on August 4, 1980 and were approved for submission for publication. The papers have been accepted for publication and are as follows:

1) A. Richters
   Facilitation of Cancer Metastases by an Air Pollutant
   Journal of Surgical Oncology 17: 159-162, 1981.

2) A. Richters and Kestutis V. Kuraitis
   Inhalation of NO_2 Blood Borne Cancer Cell Spread to the Lungs
   Archives of Environmental Health 36: 36-39, 1981.

3) Kestutis V. Kuraitis, Arnis Richters and Russell P. Sherwin
   Spleen Changes in Animals Inhaling Ambient Levels (0.35 ppm) of Nitrogen Dioxide (NO_2)
   Journal of Toxicology and Environmental Health 7: 851, 1981

4) A. Richters and Kestutis V. Kuraitis
   Air Pollutants and the Facilitation of Cancer Metastasis
   Presented at the AAAS, Pacific Division Symposium entitled: The Health Issues in Air Quality Control. Eugene, Oregon, June 15, 1981.

11. APPENDICES

Copies of the manuscripts are included as Appendix A, B, C and the presentation as Appendix D.

Inhalation of NO_2 and Blood Borne Cancer Cell Spread to the Lungs

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The authors wish to acknowledge the assistance of Drs. Russell P. Sherwin and Valda Richters.

Key Words: Nitrogen dioxide inhalation, cancer metastasis, lung.

Abstract

An experimental model was designed where the frequency of blood borne cancer cell metastases to the lungs of animals was used as an indicator for adverse effects of inhaled nitrogen dioxide (NO_2). Animals were exposed to air containing 0.40 ± 0.05 ppm or 0.80 ± 0.05 ppm of NO_2. After the appropriate exposure periods, the animals were infused intravenously with B16 mouse melanoma cells. Three weeks post-infusion, the animals were killed and the lungs were examined for melanoma nodule development. The lungs of the NO_2-exposed animals contained a significantly higher number of melanoma nodules than the lungs of control animals ($p < 0.0025$). These results indicate that the inhalation of ambient or near ambient levels of NO_2 influences the metastasis of blood borne cancer cells. This raises the possibility that similar events may take place in the human population.

A number of airborne carcinogens (e.g., Benzo-a-pyrene, DDT, Vinyl Choloride, Asbestos) have been investigated for their roles in the causation of human and other cancers (1), but little attention has been given to the question of facilitating cancer cell spread or metastasis by noxious air pollutants. However, several reports have indicated that the inhalation of nitrogen dioxide (NO_2), a common air pollutant, at ambient or near ambient levels results in functional and structural alterations in the lungs (2,3). More recently, the effects of NO_2 on the defense system of the body have been noted also (4). Pertinent to this study are the reports describing alterations in pulmonary vascular and defense systems since both of these are closely associated with blood borne cancer cell spread or metastasis. Thus, it is of interest to determine if inhalation of ambient or near ambient levels of NO_2 could facilitate the metastasis of blood borne cancer cells in an experimental model system. Moreover, could this model then be used for detecting harmful and biologically significant NO_2 effects? To test this possibility experiments were designed utilizing B16 mouse melanoma cells. B16 cells can be grown in vitro and then easily introduced into the blood stream of an animal with the eventual development of cancer nodules or metastases in the lungs.

Materials and Methods. Animal Exposure to NO_2.

Two experiments designated MR1 and M128 were carried out. Experiment MR1 was preliminary in nature and utilized 24 Swiss-Webster male mice. Experiment M128 utilized 90 C57BL/6J male mice. In experiment MR1, the animals were divided into a control and an exposed group, while in experiment M128 there were three groups - an exposed group, a control group, and a room air control group. The control and the exposed animals were housed in identical environmental chambers having a common filtered (Purafil) air intake. The desired concentration of nitrogen dioxide gas was introduced into the exposure chamber via the air intake by a method which has been described previously (5). The level of the gas

was continuously monitored with Teco chemiluminescence NO_2 analyzer and a Beckman analyzer utilizing Saltzman fluid. In addition, at least two weekly NO_2 gas level checks were performed with a fritted bubbler, employing the technique of Saltzman (5). Experiment MR1 was carried out at a NO_2 gas level of 0.4 ± 0.05 parts per million (ppm) and Experiment M128 at a level of 0.8 ± 0.05 ppm. The NO_2 gas was delivered to the exposure chambers for eight hours per day, five days per week for a total of ten weeks in experiment MR1 and twelve weeks in experiment M128.

Melanoma Cell Infusion:

B16 Melanoma Cell line F10 was received from Dr. I. Fidler and carried in this laboratory according to his protocol (6). The other B16 melanoma cell line was derived from melanoma nodules of lungs from the animal which was injected with the F10 cell line. The nodules were cultured and the derived melanoma cell line was designated "B16F10R1." All tissue cultures were maintained in plastic tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal calf serum. For experimental purposes the cells were collected from the flasks using calcium and magnesium free Earle's balanced salt solution (EBSS) containing 0.25% trypsin and 0.25% EDTA. The single cell suspensions were then washed with culture medium and resuspended in calcium and magnesium free EBSS. In experiment MR1 the B16F10 cell line was used and 10^5 cells in a volume of 0.2ml were infused via tail vein injections in all experimental and control animals. In experiment M128, cell line B16F10R1 was used and 5×10^4 melanoma cells were infused into each animal of each group by the same method.

Evaluation of Melanoma Nodules in the Lungs:

Following the prescribed exposure periods the animals were killed by an overdose of phenobarbital, the lungs were removed and inflated with 10% acetate-buffered formalin and stored in the same. The melanoma nodules visible on the surface of each lobe were counted by routine stereoscopic methodology. Sections

of the lungs have shown that almost all of the nodules are sufficiently subpleural in location for them to be easily recognized by stereoscopic methodology. The collected data was then analyzed by the Students t-test and two factor analysis of variance.

Results:

The results of experiment MR1 are summarized and presented in Table 1. The data indicate that the exposed animals developed a significantly higher number of melanoma nodules in their lungs than do the control animals at 21 days post melanoma cell infusion ($p < 0.001$). The details of the two factor analysis of variance are presented in Table 2. In addition, the nodules found in the exposed animals were larger ($p < 0.005$) after the nodules were separated into two categories, those greater and those less than 1mm in diameter.

A summary of the results for experiment M128 is presented in Table 3. The NO_2-exposed animals show a higher frequency of melanoma nodules per lung (19.0) than do the controls (7.4). The Student t-test analysis shows that the difference is highly significant with $p < 0.0025$. In comparing the room control group with exposed animals the difference is also significant and the exposed animals show higher frequency of nodules per lung with $p < 0.025$. In comparing the two control groups (C vs. RC) the animals inhaling ambient air show a higher frequency of melanoma ($p < 0.05$).

Discussion:

The results from the two experiments described in this report demonstrate that inhalation of ambient and near ambient levels of NO_2 increases the frequency of melanoma nodule development in the lungs from blood borne cancer cells. This is the first report where NO_2 is implicated as a facilitating agent for blood borne cancer cell metastasis. Moreover, this model may serve as a sensitive indicator for harmful NO_2 effects.

It is of great interest that the ambient air group (RC) of animals developed more melanoma nodules than the filtered air control. This could be in part related to the fact that our environmental chamber filters take out particulates as well as NO_x and ozone while this is not the case with regular air conditioner filters. The composition of the ambient room air could be quite complex. Spot checks for vivaria room NO_2 levels have not revealed ambient room NO_2 levels higher than 0.06 ppm. However, the NO_2 hourly maxium levels for the outside air, during this experimental period, have varied between 0.01 ppm to 0.2 ppm according to the South Coast Air Quality Management District records. The only conclusion that one can make at this time is that the ambient room air in vivarium during the experiment period enhanced the blood borne melanoma cell metastasis. The latter observation still needs confirmation.

No attempt was made to determine the mechanisms responsible for the enhancement of metastasis, but there are several obvious conditions, subsequent to NO_2 inhalation, which may contribute to blood borne cancer cell dissemination. The most likely events which may play a significant role in cancer cell spread to the lungs are the following: 1) increased pulmonary capillary permeability and endothelial cell injury; 2) suppression of host defense systems; and 3) alterations in clotting mechanisms. Whether it is a single event or a combination of the events remains to be seen. The findings, while in need of confirmation, clearly indicate that the question of facilitation of metastasis by NO_2 exposure in vivo warrants an extended investigation, not only of NO_2 itself but of other air pollutants singly and in combination. It is particularly significant that the findings have shown differences at ambient or near ambient levels of NO_2 exposure. Thus the model described may be useful for detecting subtle, harmful alterations associated with inhalation of air pollutants. It should be noted that the present California air quality standard is a one hour maximum peak level

of 0.25 ppm NO_2 and that this level is frequently exceeded in many cities throughout the United States, especially in Los Angeles. Furthermore, it raises the possibility that the inhalation of NO_2 from ambient air may facilitate the seeding and proliferation of blood borne cancer cells in the human lung.

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Table 1

Frequency of Melanoma Nodules in Lungs

Exp. MR1

Time in Days After Melanoma Cell Infusion	Treatment	No. Animals	No. Lobes	Mean No. Melanoma Nodules per Lobe (Range)	Student t-Test
10	C	5	25	5.88 (0-19)	
					$p < .05$
10	X	5	22	12.95 (0-56)	
21	C	8	40	3.42 (0-15)	
					$p < .005$
21	X	6	30	10.30 (0-47)	

X - NO_2 exposed animals

C - Control animals

Table 2

Two Factor Analysis of Variance

Exp. MR1

Source	Degrees of Freedom	Mean Square	F Ratio	p Value
Treatment (T)	1	1353.314	12.045	$p < .001$
Duration (D)	1	181.568	1.616	$p > .10$
Interaction (TXD)	1	.273	.002	NS
Residual	113	112.351	---	---

T - NO_2 exposure vs. control

D - 10 day vs. 21 days post cell infusion

Table 3

Frequency of Melanoma Nodules per Lung

Exp. M128

Treatment Groups	No. Animals	Mean No. Melanoma Nodules per Lung (Range)	Student t-test
C	23	7.4 (1-17)	C vs. X $p < 0.0025$
X	24	19.0 (2-63)	RC vs. X $p < 0.025$
RC	19	10.0 (1-26)	C vs. RC $p < 0.05$

C - Control group

RC- Room air group

X - NO_2 Exposed group

Facilitation of Cancer Metastases by an Air Pollutant

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Abstract

An experimental model was designed to test the possibility that inhalation of a noxious air pollutant may facilitate the blood-borne cancer cell metastasis to the lungs. Animals were exposed to inhalation of air containing 0.8 ppm of nitrogen dioxide for 12 weeks. After this period animals were infused intravenously with melanoma cells and 3 weeks later lungs were examined for metastases. The results indicate that NO_2 exposed animals develop significantly higher number of lung metastases ($p < 0.0025$) than the controls. Such results raise the possibility that the inhalation of NO_2 from ambient air may facilitate the seeding and proliferation of blood-borne cancer cells in the human lung.

Key Words: Nitrogen dioxide inhalation, cancer metastasis, lung

INTRODUCTION:

The problem of cancer involves not only the development of a cancer at a primary site but the seeding and proliferation of the neoplastic cells in distant organs and tissues. This important principle has recently been emphasized by Day, who stated that "even though the cause of cancer is important, in the clinical case it is the spread - the phenomenon of metastasis- that is of much more immediate concern in the human situation" [1]. It is well recognized that the presence of cancer cells in the circulation is not invariably associated with the development of metastasis [2,3]. It also is known that the successful lodgment of cancer cells and their subsequent proliferation is dependent upon a number of factors other than the nature of the cancer cells themselves. The more important of these factors are the host defense system, capillary endothelial integrity, the clotting mechanisms and cell injury in general [4,5,6]. Thus, the agents that injure the lung may also increase the susceptibility of the lung to cancer metastasis. A likely candidate to cause such injury is a noxious air pollutant that can reach the pulmonary acini. Nitrogen dioxide (NO_2), a common air pollutant, has been demonstrated to produce detectable structural and functional changes in the lungs when inhaled at ambient and near ambient levels [7,8,9]. Our preliminary experiments suggest that NO_2 exposure may enhance cancer metastasis [10]. The findings presented in this report provide additional information that the inhalation of NO_2 promotes metastasis to the lungs from blood borne cancer cells in an experimental model system.

MATERIALS AND METHODS:

An experiment was designed to test the effects of NO_2 on the frequency of the development of pulmonary metastasis from blood borne mouse melanoma cells (B16 F10 R1) in C57 B1/J6 mice. The cell line B16 F1D R1 was derived from a metastatic nodule in a lung, which had developed from injection of

B16 F10 [11] cell line. A total of 60 mice were studied. Animals were divided into two equal groups of 30 animals each, and were housed in environmental chambers. The control animals received filtered, NO_2-free air, while the experimental group was exposed to air containing 0.8 ppm NO_2. NO_2 was delivered to the exposure chamber by a method described previously [12] and the NO_2 level was monitored by the Saltzman method [13] and a chemiluminescence detector. Animals were exposed 7 hours per day, 5 days per week for a total of 12 weeks. At this time both control and experimental animals were infused with 50,000 B16 F10 R1 melanoma cells via the lateral tail vein and housed in ambient vivaria air. Twenty-one days post infusion the animals were killed, the lungs were removed en bloc and inflated with 10% acetate buffered formalin and evaluated for melanoma nodules. The melanoma nodules (pigmented) were counted with respect to each lobe of the lung, utilizing the stereomicroscope. The melanoma nodules in most cases are located on the subpleural surfaces and are easily detectable by this methodology. Histological sectioning of lungs revealed that only a few nodules are located in the interior of the lung parenchyma. The collected data was then analyzed utilizing the Students t-test.

RESULTS:

During the course of the experiment, some animals were lost due to causes unrelated to the experiment and the results are based on 23 control and 24 exposed animals. There was a considerable variation from animal to animal in the number of nodules which developed in the lungs in both control and experimental groups. The size of the nodules lso varied and ranged from a fraction of a millimeter to several millimeters in diameter. The most significant finding is reflected in the total number of nodules per lung, with the lungs of the NO_2- exposed animals showing a significantly higher

number of melanoma nodules ($p < 0.0025$) (Table 1). The distribution of nodules is presented in Table 2 and the left and right upper lobes show the highest frequency. With respect to metastasis to other sites, only liver metastases were noted with 17% of the control animals and 41% of the NO_2-exposed animals showing liver metastasis.

DISCUSSION:

The findings in this study confirm our preliminary observations and clearly indicate that the question of facilitation of metastasis by NO_2 exposure in vivo warrants an extended investigation, not only of NO_2 itself but of other air pollutants as well. It is particularly significant that the findings have shown differences at both ambient (0.4 ppm) and near ambient (0.2 ppm) NO_2 levels. It should be noted that the present California air standard is one hour maximum peak level of 0.25 ppm NO_2 and that this level is frequently exceeded in many cities throughout the United States. Thus, of particular significance to community health is the possibility that the inhalation of NO_2 at ambient levels may facilitate the seeding and growth of blood borne cancer cells in the human lung. At the present time there is no information available with respect to such a human experience. Therefore, epidemiological studies correlating the recurrence of metastasis of cancer with a patient's post-operative environmental experience would be of great interest. As indicated earlier, metastasis depends upon many intrinsic factors in an individual, but very little is known about the extrinsic environmental factors that may affect metastasis. It should be noted that inhalation of cigarette smoke also has been associated with enhanced metastasis in another experimental system [14]. Thus, by utilizing the experimental model described in this study, together with other approaches, one should achieve better understanding of the relationship between inhalation of air pollutants and the process of cancer.

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Table 1

Frequency of Melanoma Nodules in Lung

Treatment Groups	No. Animals	Total No. Nodules	Mean No. Nodules per lung	Student t-test
C	23	172	7.4	
				$p < .0.0025$
X	24	449	18.7	

C - Control

X - NO_2 Exposed

Table 2

Distribution of Melanoma Nodules

Treatment Group	No. Animals	Nodules per lobe*					Total No. Nodules
		L	RU	RM	RL	C	
Control	23	52	33	30	40	17	172
NO_2 Exposed	24	115	121	67	94	52	449

* L Left lobe

RU Right upper lobe

RM Right middle lobe

RL Right lower lobe

C Cardiac lobe

SPLEEN CHANGES IN ANIMALS INHALING AMBIENT LEVELS (0.35 ppm) OF NITROGEN DIOXIDE (NO_2)

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ABSTRACT

The effects of ambient levels of nitrogen dioxide (NO_2) on the spleens of adult and newborn Swiss Webster (S/W) mice were determined. The spleens were evaluated by the following criteria: 1) spleen weights, expressed as a percent of the body weight (% spleen weight), 2) size of the spleen lymphoid nodules as determined by computed image analysis, 3) spleen cell counts, and 4) histopathologic evaluation. All the data obtained for the NO_2-exposed animals was compared to control animals that inhaled filtered air. A total of 217 control and 217 NO_2-exposed animals were studied. After 6 weeks of ambient (0.35 ± 0.05 ppm) level NO_2 exposure, the following significant spleen changes were observed in the exposed group of mice: 1) increase in the percent (%) spleen weights ($p < .0025$), 2) increase in the size of the spleen lymphoid nodules ($p < .01$), 3) smaller increase in spleen cell numbers per given weight increment of spleen as determined by correlation coefficients ($p < .0125$) and linear regression analysis of spleen cell counts, and 4) although not quantitatively determined, there appeared to be a greater predominance of red cells in the red pulp. It is concluded from this study that the inhalation of NO_2 is associated with quantifiable spleen changes which may prove to be useful indicators for assessing effects of inhaled NO_2.

INTRODUCTION

Nitrogen dioxide (NO_2) is one of the major air pollutants frequently encountered in the urban environment. Moreover, it has been shown that the inhalation of NO_2 can produce both functional and structural changes in the lungs of experimental animals. The changes have been associated with both high and ambient level NO_2 exposures, and for many years investigations on the adverse effects of NO_2 have focused primarily on the lung (Dawson and Schenker, 1979; Guidotti, 1978). Very few studies have focused attention on other organs (Ehrman, et al., 1972; Sherwin and Layfield, 1974), in spite of indications that harmful NO_2 reaction products may be distributed systemically and may be capable of reacting with particular blood elements (Case, et al., 1979; Oda, et al., 1980). Considering these observations together with the fact that the spleen is closely interrelated with circulating blood components, the question arises as to whether the spleen is physiologically or pathologically responding to the inhalation of NO_2. In addition, data from earlier experiments in our laboratory (unpublished observations) indicated that a difference in spleen size existed between control and NO_2 exposed animals. Thus, observations of spleen changes were incorporated into the protocol of all subsequent NO_2 inhalation studies. This report covers three experiments with a total of 434 mice, half of which were exposed to 0.35 ppm NO_2 for a period of six weeks.

The spleen is the largest lymphatic organ of the immune system and plays a prominent role in host immune responses. Moreover, there are recent reports that the spleen may play an important role in modulating

local pulmonary immune responses (Liu and Plautt, 1980; Stein-Streilein, et al., 1979). The latter authors have demonstrated that both humoral and cell-mediated pulmonary immune reactions are influenced by splenic responses. Thus with respect to the inhalation of NO_2 the spleen changes described in this report may become very important in understanding the toxic effects on pulmonary tissues and the defense system. At the present time, very limited information exists with respect to the inhalation of air pollutants and spleen lymphoid cell responses. It has been reported that the inhalation of NO_2 depressed the responses of splenic T and B cells to mitogenic stimuli (Maigetter, et al., 1978). Another more recent study on a variety of immune parameters, such as graft rejection, immunoglobulin production, etc., reported a suppression of these immune functions with chronic exposures and an actual enhancement with shorter exposure periods (Holt, et al., 1979). The study reported here shows a definite relationship in that the inhalation of ambient level NO_2 is associated with spleen weight and cellular changes. This strongly suggests that in addition to lung changes, the inhalation of ambient level NO_2 is associated with quantifiable changes in the spleen.

METHODS

Animal Exposure to NO_2

In each of the experiments, the animals were divided equally into two groups and housed in identical environmental chambers having a common filtered air intake. A predetermined concentration of nitrogen dioxide gas was supplied to the air inflow of the experimental chamber, details of which have been described previously (Sherwin & Yuen, 1972). The level of the gas was continuously minitored with a Teco chemiluminescence NO_2 analyzer and a Beckman analyzer using Saltzman fluid. In addition, at least two weekly NO_2 gas level checks were performed with a fritted bubbler, employing the technique of Saltzman (Saltzman, 1954). The experiments described in this paper were conducted at an NO_2 gas level of 0.30 ± 0.05 ppm. In addition, fritted bubbler samples were taken from the control chamber. NO_2 gas levels in these chambers never exceeded 0.02 ppm. The NO_2 gas was delivered to the experimental chamber for eight hours per day, five days per week, for a total of six weeks. Three separate experiments, designated M117, M119 and M123 were carried out and the effects of NO_2 on lungs and spleens were studied. Experiment M119 differed slightly from the other two in that the animals were injected intravenously with horseradish peroxidase (HRP) three and a half hours prior to animal killing. The HRP injection was part of another experiment on the same group of animals. In experiments M117 and 119, pregnant female mice were placed into the chambers two weeks prior to delivery and the pups were born in the chambers. A total of 134 male newborn and 300 male adult Swiss Webster mice were studied. This report covers only the studies dealing with spleen changes and body weight changes.

Spleen Weight Assay

Animals were kept in the environmental chambers for six weeks. After this time, equal numbers of control and exposed animals were removed from the respective chambers, the body weights were recorded and the mice were then killed in a sequential order of matched control and exposed animal pairs with a 0.5 cc intraperitoneal injection of sodium pentobarbital. The animals were exsanquinated by cutting the abdominal aorta and carotids. The spleens were then removed and any adherent tissue was trimmed off. The spleens were weighed immediately and the weights were expressed as a percent of the body weight (% spleen weight). The reproducibility of wet spleen weights was very good.

Computed Image Analysis of Spleen Lymphoid Nodules

Computed Image Analysis (IA) was carried out on 30 spleens from S/W newborn mice (6 weeks old). Spleens were fixed in 10% sodium acetate buffered formalin, and embedded in paraffin. Four sections at 150 micron intervals were cut from each spleen to obtain more representative cross sections of the lymphoid nodules. Ten micron sections were then stained with hematoxylin only, since this stain alone provided the needed contrast for IA. IA was performed with the Quantimet 720 on two nonoverlapping areas from each spleen section and with four sections per spleen, provided a total of eight independent area measurements. The following three measurements were performed in each field: 1) Total Nodular Area (A_{nod}); 2) Total Spleen Area (A_{spl}); and 3) Number of Nodules (#nod). The measurements were expressed as A_{nod}/A_{spl}, $A_{nod}/\#nod$ and $\#nod/A_{spl}$ to determine the mean area of nodules per given area of spleen,

the mean nodule size and the number of nodules per given area of spleen, respectively. Spleen sections were also processed for histopathologic evaluation.

Spleen Cell Evaluation

Spleen cell suspensions were prepared by standard techniques from the three lightest and heaviest spleens from both experimental and control groups in Exp. 119. In brief, spleens were finely cut, suspended in calcium and magnesium free Earl's balanced salt solution (EBSS) supplemented with 5% heat-inactived fetal calf serum, and gently put through a stainless stell mesh with a rubber policeman. The cell suspensions were centrifuged at 400 x g for 10 minutes at 4°C. The resulting pellet was resuspended in eight volumes of 0.83% ammonium chloride (NH_4Cl) for 10 minutes to lyse the red blood cells. The cells were then washed as before, resuspended in medium and incubated for two hours at 37°C to permit the attachment of adherent cells. The nonadherent cells were then removed, referred to as "splenocytes", and counted with the Coulter counter. The data from the spleen assays and IA was analyzed utilizing the Student's t-test. Correlation coefficients and a linear regression plot were used to analyze the splenocyte number data.

RESULTS

Percent Spleen Weights

It can be seen from the data in Figure 1 that % spleen weights of NO_2-exposed animals were significantly greater in animals exposed to ambient levels of NO_2. In experiments M123, the p value approached zero, while in experiments M117 and M119, p was less than .0025 and .0005 respectively.

However, in the group of animals receiving the i.v. injections of HRP, there was a greater variation in the % spleen weights from the mean value in both the control and the experimental group. The overall ratio values were also the highest in this group of animals. In addition, the data presented in Table 1 demonstrates that the absolute spleen weights were significantly greater in the NO_2 exposed mice in all three experiments. With respect to body weights, experiment M119 demonstrated a significant decrease in the mice exposed to NO_2.

Splenocyte Numbers

Splenocyte numbers in the spleens correlated with the spleen weights as well as with % spleen weights (Table 2). The control animals demonstrated a more significant correlation coefficient. Moreover, the linear regression plot of the data indicates that the exposed animal spleens should have a smaller increase in splenocyte number per a given weight increment of spleen (Figure 2).

Image Analysis (IA) of Spleens

IA was performed on the spleens of 30 mice from experient M117 and included a total of 103 fields in the control group and 97 fields in the exposed: The data, presented in Table 2, demonstrates that the NO_2-exposed group had an increase in the mean nodule size. When the mean lymphoid nodule area of the exposed group was expressed as a ratio to a given unit area of spleen, the results were significant in the upper and lower quartiles. This data points out the fact that the non-lymphoid regions (red pulp) in the spleens of NO_2-exposed animals increased in area

as well. With respect to germinal centers, no measurements were made.

Microscopic Observations of Spleens

Microscopic examination of H & E sections of the spleens have revealed that in most of the control animals the red pulp area frequently contains hematopoietic elements while in the exposed animals, the red pulp area appears to have less hematopoietic elements and more erythrocytes. Histopathologic evaluation of the sections did not reveal any apparent differences in germinal center morphology.

DISCUSSION

This study demonstrates that the inhalation of ambient levels of NO_2 (0.35 ± 0.05 ppm) for periods of 6 weeks resulted in changes of spleen weight, body weight, and the size of spleen lymphoid nodules. In addition, the studies on splenocytes, even though of a more limited scope, indicated that the NO_2-exposed spleens had a decrease in the number of splenocytes per given weight of spleen.

In order to explain this observation one must consider several mechanisms. First, it is quite possible that due to the variety of NO_2 induced pulmonary changes, the lung may become a more favorable environment for microorganisms. Thus, the immune response to this increased microbial challenge, especially if viral in nature, could be responsible for lymphoid nodule changes. Second, the spleen itself may undergo changes similar to that of the lung and the shift in spleen weight may result from a splenic reaction to some type of local alteration

directly induced by NO_2 or its reaction products. Third, since NO_2 or its reaction products have been shown to induce lipid peroxidation (Thomas, et al., 1968; Roehm, et al., 1971), circulating erythrocytes and leucocytes may incur membrane damage. This increased damage to blood cells could increase phagocytic activity or macrophage numbers in the spleen and contribute to spleen weight changes.

With respect to spleen cellularity, the observation that the red pulp area increases in exposed animals, could mean an increase in erythrocyte numbers, but this study did not quantitatively evaluate this observation. Other reasons could, and probably do, exist for explaining some of the spleen changes, and more experiments will be needed to elucidate the mechanisms involved. Regardless of the mechanism involved, the following conclusions can be made from this study:

1) % spleen weight, when compared between NO_2-exposed and control animals, provides a simple and reproducible indicator for ambient level NO_2 inhalation exposure; 2) The inhalation of ambient level NO_2 is associated with the enlargement of splenic lymphoid nodules; 3) The observed body and spleen weight differences between the exposed and control animals may be indicative of systemic NO_2 effects; and 4) The spleen weight assay described in this report could provide a new and simple approach for assessing air quality. Finally, it should be pointed out that preliminary observations along with current experiments are beginning to suggest that these observed spleen changes may depend upon the level of NO_2 as well as the length of exposure (Kuraitis, 1979; 1980). It appears that at ambient levels of NO_2 exposure for six weeks, % spleen

weights are higher in the animals exposed to the NO_2, while at 12 weeks exposure the % spleen weight is lower in the exposed group. The latter results are preliminary and need further confirmation, but they do point to the possibility of a biphasic spleen response. This would be supportive of the results reported by Holt (Holt, et al., 1979).

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TABLE 1 - SPLEEN WEIGHTS AND BODY WEIGHTS AFTER 6 WEEKS NO_2 EXPOSURE (0.35 ± 0.05 ppm)

Experiment No.	Animal No.	Body Weight (grams) ± S.D.			Spleen Weight (mg) ± S.D.		
		Control	Exposed	P^a	Control	Exposed	P^a
M117	30 Newborn	25.22±3.82	24.36±3.85	N.S.	121.85±30.11	143.32±21.73	<.025
M119	104 Newborn	25.13±3.54	23.42±4.62	<.025	155.36±27.70	166.22±38.11	<.05
M123	300 Adults	32.60±2.46	32.53±2.64	N.S.	102.99±16.25	123.14±17.49	→0

P^a - probability values

TABLE 2 - CORRELATION COEFFICIENTS (r) FOR SPLEEN WEIGHTS AND PERCENT (%)

SPLEEN WEIGHT VS NUMBER OF SPLENOCYTES

	Spleen Wt. vs No. of Splenocytes			Percent % Spleen Wt. vs No. of Splenocytes		
No. of Animals	r	t-value[a]	P[b]	r	t-value[a]	P[b]
6 Control	.974	8.54	$\leq .0025$	.969	7.85	$< .0025$
6 Exposed	.879	3.68	$< .0125$	.894	3.99	$< .01$

P[a] - $t = \sqrt{\nu r^2/(1-r^2)}$, where ν = degrees of freedom (n-2), r = correlation coefficient

P[b] - probability

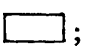
TABLE 3 - COMPUTED IMAGE ANALYSIS OF SPLEEN LYMPHOID NODULES IN CONTROL AND NO_2-EXPOSED ANIMALS

Spleen Measurements[a]		Control ± S.D.	Exposed ± S.D.	P^b
Nodule Area / Given Spleen Area	All Data	.147±.047	.156±.048	< .1
	Upper Quartile	.203±.025	.221±.025	< .01
	Lower Quartile	.087±.023	.097±.017	< .05
No. of Nodules Per Given Area of Spleen		4.00±1.5	3.47±0.9	< .0025
Mean Nodule Size		416.23±215.49	498.95±254.59	< .01

P^a - All measurements are in quantimet units

P^b - Probability value

Figure 1 Bar graph comparing the percent spleen weight with standard deviations ($\pm$S.D.) of control and NO_2-exposed animals from three separate experiments. Animal numbers used are in parentheses.

(Control  ; NO_2-exposed )

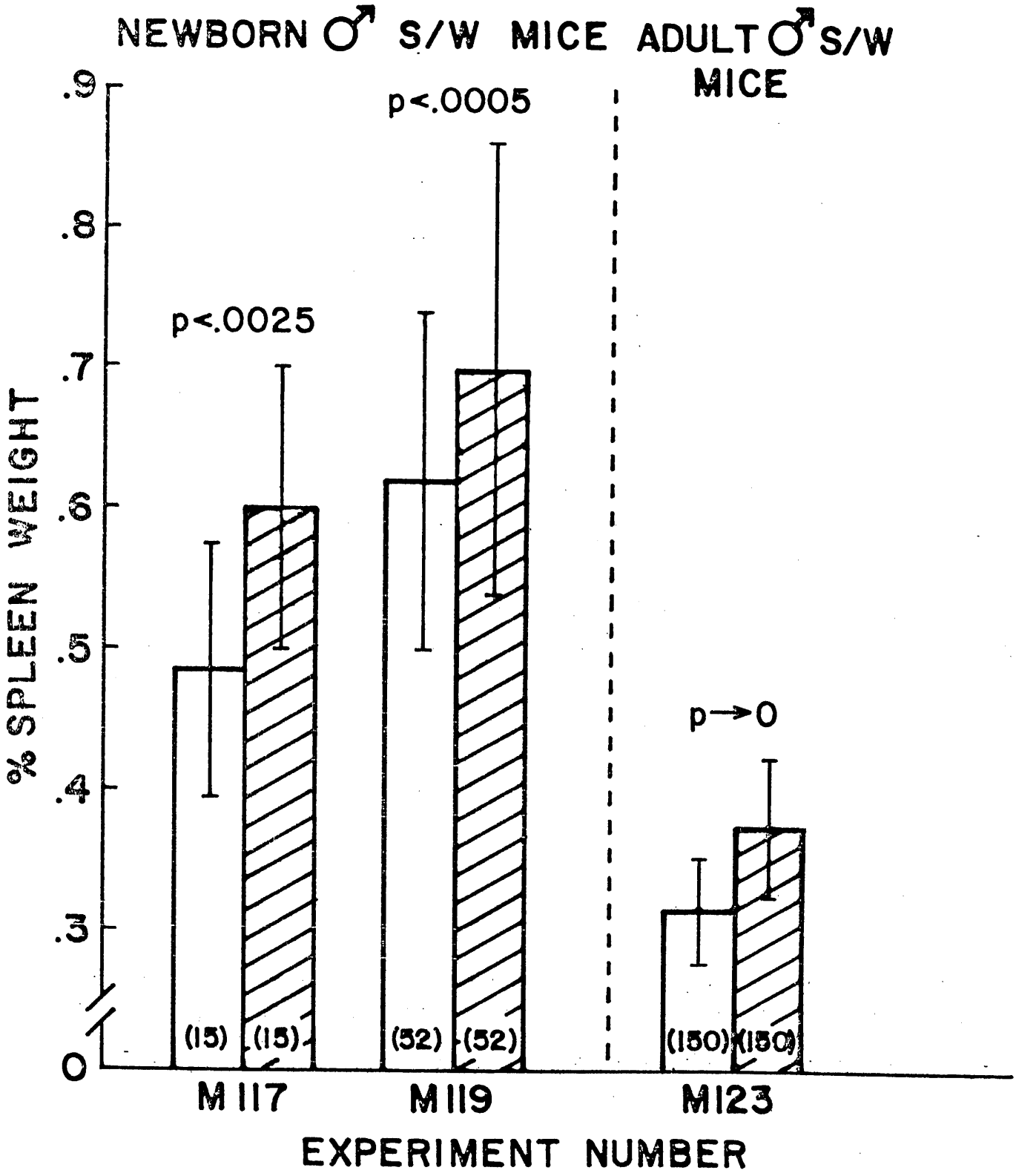
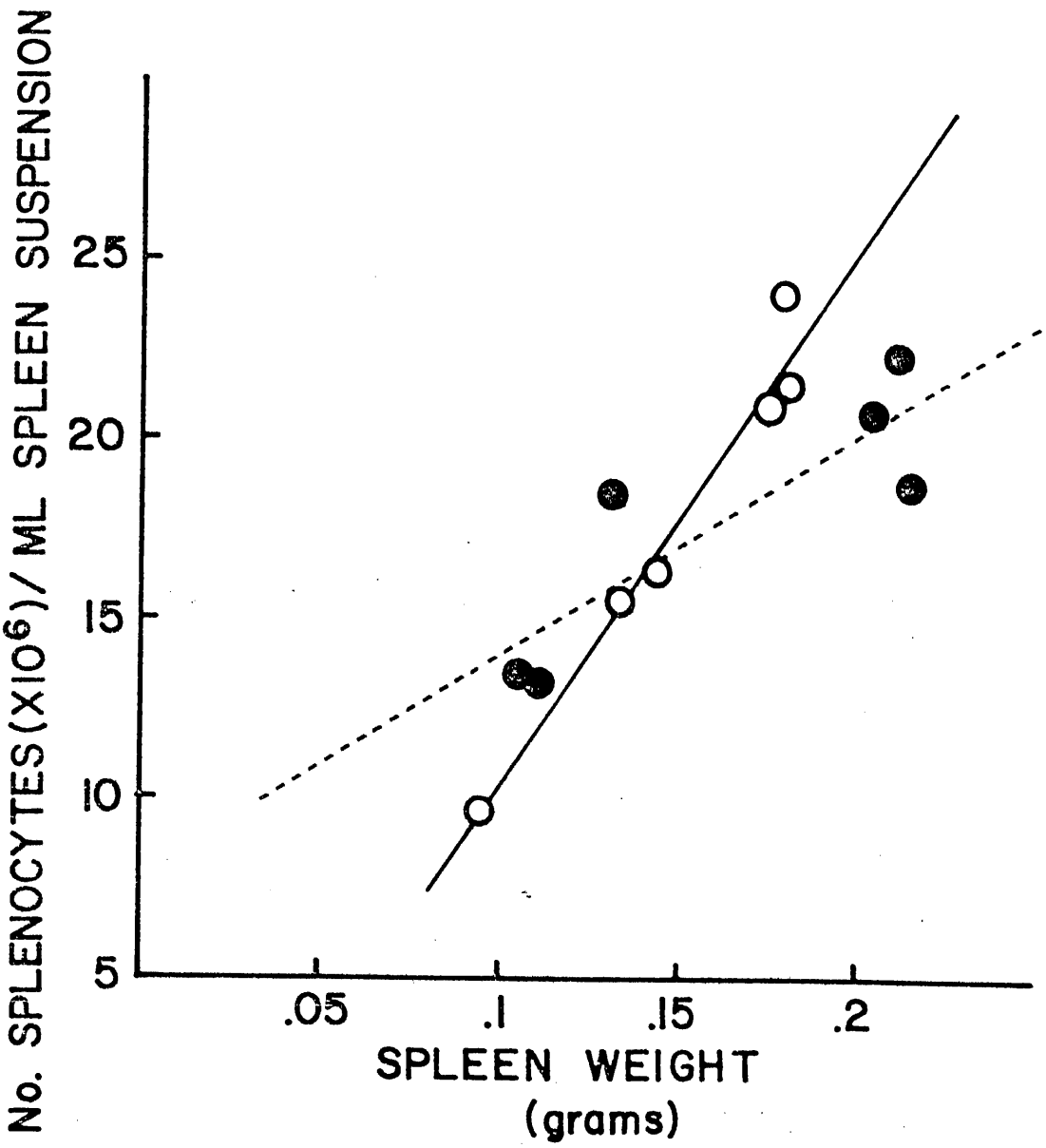

NEWBORN ♂ S/W MICE
ADULT ♂ S/W MICE
p<.0005
p<.0025
p→0
% SPLEEN WEIGHT
.9
.8
.7
.6
.5
.4
.3
0
(15)
(15)
(52)
(52)
(150)
(150)
M117
M119
M123
EXPERIMENT NUMBER

Figure 2 Linear regression plot comparing the number of splenocytes against spleen weights in control and NO_2-exposed mice. (Control—○—○—; NO_2-exposed --●--●--)


No. SPLENOCYTES (X10⁶) / ML SPLEEN SUSPENSION
25
20
15
10
5
.05
.1
.15
.2
SPLEEN WEIGHT
(grams)

"AIR POLLUTANTS AND THE FACILITATION OF CANCER METASTASIS"

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ABSTRACT

Studies have been carried out to determine whether the inhalation of ambient levels of nitrogen dioxide (NO_2), a common air pollutant, could influence the frequency of blood borne cancer cell metastasis to the lungs. B16 mouse melanoma cells were used as an in vivo test model. The results have indicated that animals inhaling ambient levels of NO_2 developed a significantly higher number of melanoma nodules in their lungs than the animals inhaling filtered air. The question is raised whether similar events are taking place in the urban human population.

The presence of pollutants in the environment, especially those with carcinogenic properties, has been of great concern to environmental health scientists. In view of this, many studies have been directed toward the identification of cancer causing agents (1,2). However, the problem of cancer involves not only the development and presence of neoplastic cells at a primary site, but also the ability of these cells to migrate, seed, and proliferate in distant organs and tissues. The importance of cancer cell dissemination and metastasis has been emphasized by many investigators and has been stated particularly well by Day, who wrote that, "even though the cause of cancer is important, in the clinical case it is the spread - the phenomenon of metastasis - that is of much more immediate concern in the human situation" (3). Moreover, considering the fact that a significant segment of the population is already affected by cancer together with the probability that one in four individuals will develop cancer (4), the question arises as to the role environmental pollutants play not only in the causation of cancer or carcinogenesis, but in dissemination of cancer cells and development of metastases.

Since the lung is exposed to many inhaled pollutants, a strong possibility exists that inhalation of noxious air pollutants could facilitate the development of cancer cell metastases in the lung. Development and progression of cancer is a very complex process and it is possible that different air pollutants could act at different sites in the sequence of cancer progression. In general one could say that certain air pollutants could participate in the process of carcinogenesis and others in the process of cancer cell dissemination and metastasis (Figure 1). In the first case, noxious air pollutants could act as initiators, co-carcinogens, or promoters leading

to the development of cancer. In the second case, they could act as facilitators of cancer cell dissemination by exerting their effects on the host in a non-carcinogenic manner.

It is well established that most cancer patients have circulating cancer cells (5,6) and in some instances cancer cells have been demonstrated in the circulation of patients who are clinically free of cancer (7,8). In addition, circulating cancer cells are also found in peripheral blood of tumor bearing animals (9). Moreover, there are several known conditions which may favor the development of cancer cell metastases from these circulating cells and include the following: 1) immune suppression, 2) endothelial cell alterations, 3) cancer cell homotypic or heterotypic aggregation, 4) cancer cell interactions with components of blood clotting mechanisms, and 5) tissue damage in general (10-14). It is known that several of the above mentioned conditions occur as a result of nitrogen dioxide exposure (15-18) and thus one may expect that air pollutant inhalation could facilitate or enhance circulating cancer cell metastasis to the lungs. Recent experiments in our laboratory utilizing a mouse melanoma model have indeed demonstrated that inhalation of ambient levels of NO_2 facilitates blood borne cancer cell metastasis to the lungs, (19,20) and in this report we present additional data which supports the earlier findings (Table 1). Thus, for the first time a noxious air pollutant is implicated in the facilitation of blood borne cancer cell dissemination. The mechanism involved is not clear and it is possible that several of the conditions which favor the development of cancer metastases may be involved.

It is important to point out that in our experiments the inhalation

of polluted urban ambient air produced the same effects as the inhalation of 0.3 ppm NO_2 for 12 weeks (Table 1, Experiment 137). The inhalation of a slightly higher concentration of NO_2 (0.5 ppm) for a period of 8 weeks did not have the same effect (Experiment 139) and to date only exposures of 10 weeks or longer to ambient concentrations of NO_2 have enhanced the development of metastases in lungs. If we consider these findings in light of our spleen studies (21,22) it is possible that the immune status of the exposed animals is different following short and prolonged ambient level NO_2 exposures. The same or similar dependence on exposure length is probably involved in other ambient level NO_2 effects as well.

With respect to human cancer, epidemiological studies have shown increased mortality rates from cancers in polluted urban areas (23,24), however, other reports indicating no increases also exist (25,26). Epidemiological studies designed to study the metastasis question specifically are missing and several existing reports often equate the increased incidence of cancer with an increased incidence of mortality and thus present problems with interpretation. Epidemiological studies where the frequency of cancer metastases development could be correlated specifically with environmental exposures are needed urgently. Moreover, even though the data on the subject of blood borne cancer cell metastasis and air pollution are limited and come from experimental animal studies, we consider these findings highly relevant to human health and to the setting of air quality standards.

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LEGENDS

FIGURE 1 Action of air pollutants on the process of carcinogenesis and on the facilitation of cancer cell metastasis.

TABLE 1 Frequency of pulmonary metastases.

FIGURE 1

ACTION OF AIR POLLUTANTS

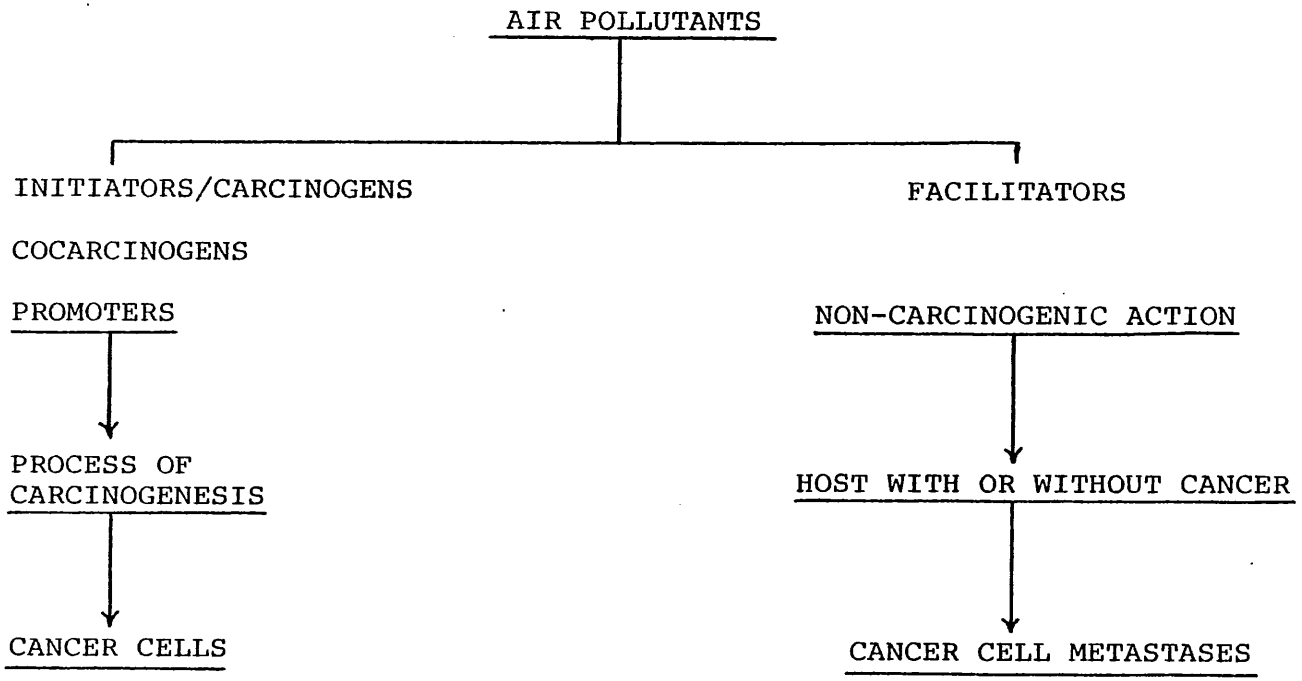

TABLE 1

FREQUENCY OF PULMONARY METASTASES

EXP.	TREATMENT	NO. ANIMALS	MEAN NO. NODULES PER LUNG	P VALUE
M128	FILTERED AIR	23	7.5	P = 0.0004
12 WEEKS	NO_2; 0.8 PPM	24	19.0	
	AMBIENT AIR	19	10.8	NS
M137	FILTERED AIR	29	10.1	P = 0.0542
12 WEEKS	NO_2; 0.3 PPM	25	15.6	
	AMBIENT AIR	28	15.1	P = 0.0305
M139	FILTERED AIR	29	10.7	NS
8 WEEKS	NO_2; 0.5 PPM	23	9.3	
	AMBIENT AIR	26	10.7	NS

P = Wilcoxon 2-Sample Test